Amendments to Specification

Title at page 1, lin 2:

ISOLATED NUCLEIC ACIDS ENCODING METHYLENETETRAHYDROFOLATE DEHYDROGENASE ~~TETRAHYDROFOLATE METABOLISM~~ ENZYMES

Paragraph on page 1, lines 3-4;

This application is a divisional of U.S. Patent Application No. 09/903,814, filed July 12, 2001, still pending, which is a divisional of U.S. Patent Application No. 09/351,703, filed July 12, 1999, now abandoned, which claims the benefit of U.S. Provisional Application No. 60/092,869, filed July 15, 1998, now abandoned.

Paragraph beginning on page 7, line 36, and continuing through page 8, line 12:

Substantially similar nucleic acid fragments of the instant invention may also be characterized by the percent identity of the amino acid sequences that they encode to the amino acid sequences disclosed herein, as determined by algorithms commonly employed by those skilled in this art. Preferred are those nucleic acid fragments whose nucleotide sequences encode amino acid sequences that are 80% identical to the amino acid sequences reported herein. More preferred nucleic acid fragments encode amino acid sequences that are 90% identical to the amino acid sequences reported herein. Most preferred are nucleic acid fragments that encode amino acid sequences that are 95% identical to the amino acid sequences reported herein. Sequence alignments and percent identity calculations were performed using the MEGALIGN® ~~Megalign~~ program of the LASERGENE® ~~LASARGENE~~ bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS. 5*:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

Paragraph at page 8, lines 13-35:

A "substantial portion" of an amino acid or nucleotide sequence comprises an amino acid or a nucleotide sequence that is sufficient to afford putative identification of the protein or gene that the amino acid or nucleotide sequence comprises. Amino acid and nucleotide sequences can be evaluated either manually by one skilled in the art, or by using computer-based sequence comparison and identification tools that employ algorithms such as BLAST (Basic Local Alignment Search Tool; Altschul et al. (1993) *J. Mol. Biol. 215*:403-410~~; see also www.ncbi.nlm.nih.gov/BLAST/~~). In

general, a sequence of ten or more contiguous amino acids or thirty or more contiguous nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene-specific oligonucleotide probes comprising 30 or more contiguous nucleotides may be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., *in situ* hybridization of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12 or more nucleotides may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises a nucleotide sequence that will afford specific identification and/or isolation of a nucleic acid fragment comprising the sequence. The instant specification teaches amino acid and nucleotide sequences encoding polypeptides that comprise one or more particular plant proteins. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a substantial portion of the disclosed sequences for purposes known to those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.

Paragraph at page 19, lines 7-22:

cDNA libraries may be prepared by any one of many methods available. For example, the cDNAs may be introduced into plasmid vectors by first preparing the cDNA libraries in <u>UNI-ZAP®</u> ~~Uni-ZAP*~~ XR vectors according to the manufacturer's protocol (<u>STRATAGENE®</u> ~~Stratagene~~ Cloning Systems<u>; Stratagene Corporation</u>, La Jolla, CA). The <u>UNI-ZAP®</u> ~~Uni-ZAP*~~ XR libraries are converted into plasmid libraries according to the protocol provided by Stratagene <u>Corporation</u>. Upon conversion, cDNA inserts will be contained in the <u>pBLUESCRIPT®</u> plasmid vector ~~pBluescript~~. In addition, the cDNAs may be introduced directly into precut <u>BLUESCRIPT®</u> ~~Bluescript~~ II SK(+) vectors (Stratagene <u>Corporation</u>) using T4 DNA ligase (New England Biolabs), followed by transfection into DH10B cells according to the manufacturer's protocol (GIBCO BRL Products). Once the cDNA inserts are in plasmid vectors, plasmid DNAs are prepared from randomly picked bacterial colonies containing recombinant <u>pBLUESCRIPT®</u> ~~pBluescript~~ plasmids, or the insert cDNA sequences are amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences. Amplified insert DNAs or plasmid DNAs are sequenced in dye-primer sequencing reactions to generate partial cDNA sequences (expressed sequence tags or "ESTs"; see Adams et al., (1991) *Science* *252*:1651). The resulting ESTs are analyzed using a <u>PERKIN-ELMER®</u> ~~Perkin Elmer~~ Model 377 fluorescent sequencer.

Paragraph beginning at page 19, line 25, and continuing through page 20, line 11:

cDNA clones encoding tetrahydrofolate metabolism enzymes were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul et al. (1993) *J. Mol. Biol. 215*:403-410~~; see also www.ncbi.nlm.nih.gov/BLAST/~~) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The cDNA sequences obtained in Example 1 were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish and States (1993) *Nature Genetics 3*:266-272) provided by the NCBI. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins.

Paragraph beginning at page 20, line 34, and continuing through page 21, line 9:

Sequence alignments and percent identity calculations were performed using the MEGALIGN™ ~~Megalign~~ program of the LASERGENE® ~~LASARGENE~~ bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS. 5*:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. Sequence alignments and BLAST scores and probabilities indicate that the nucleic acid fragments comprising the instant cDNA clones encode a substantial portion of a 3-methyl-2-oxobutanoate hydroxymethyltransferase. These sequences represent the first corn sequences encoding 3-methyl-2-oxobutanoate hydroxymethyltransferase.

Paragraph at page 22, lines 6-16:

Sequence alignments and percent identity calculations were performed using the MEGALIGN™ ~~Megalign~~ program of the LASERGENE® ~~LASARGENE~~ bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS.* *5*:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. Sequence alignments and BLAST scores and probabilities indicate that the nucleic acid fragments comprising the instant cDNA clones encode a substantial portion of a formyltetrahydrofolate deformylase. These sequences represent the first corn, rice and wheat sequences encoding formyltetrahydrofolate deformylase.

Paragraph at page 23, lines 6-16:

Sequence alignments and percent identity calculations were performed using the MEGALIGN™ ~~Megalign~~ program of the LASERGENE® ~~LASARGENE~~ bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS.* *5*:151-153) with the default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE=1 ~~KTUPLE 1~~, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. Sequence alignments and BLAST scores and probabilities indicate that the nucleic acid fragments comprising the instant cDNA clones encode a substantial portion of a glutamate formiminotransferase. These sequences represent the first rice, soybean and wheat sequences encoding glutamate formiminotransferase.

Table 9 on page 24:

TABLE 9

BLAST Results for Sequences Encoding Polypeptides Homologous to *Pisum sativum* Methylenetetrahydrofolate Dehydrogenase

Clone	Status	BLAST pLog Score to (gi 4103987 ~~1706872~~)
Contig composed of: cbn10.pk0021.f6 cbn2.pk0047.b3 cbn2n.pk0012.g11 cc71.pk0002.f2 cco1n.pk0041.d9 cen3n.pk0027.c12 cen3n. pk0128.d9 cr1n.pk0196.f11 cta1n.pk0073 g9	Contig	130.00
rlr6.pk0059.b1	EST	113.00
ses2w.pk0009.d8	EST	88.70
Contig composed of: wdk1c.pk012.f19 wl1.pk0006.h11 wle1n.pk0031.g8 wr1.pk0101.c9 wr1.pk0118.c6 wre1n.pk170.f4 wre1n.pk170.g10	Contig	82.15

Table 10 on page 24:

TABLE 10

Percent Identity of Amino Acid Sequences Deduced From the Nucleotide Sequences of cDNA Clones Encoding Polypeptides Homologous to *Pisum sativum* Methylenetetrahydrofolate Dehydrogenase

SEQ ID NO.	Percent Identity to (gi 4103987 ~~1706872~~)
16	78%
18	66%
20	63%
22	55%

Paragraph beginning at page 24, line 13, and continuing through page 25, line 4:

Sequence alignments and percent identity calculations were performed using the MEGALIGN™ ~~Megalign~~ program of the LASERGENE® ~~LASARGENE~~ bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences was performed using the Clustal method of alignment (Higgins and Sharp (1989) *CABIOS. 5*:151-153) with the default parameters (GAP PENALTY=10,

GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method were KTUPLE=1 ~~KTUPLE 1~~, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. Sequence alignments and BLAST scores and probabilities indicate that the nucleic acid fragments comprising the instant cDNA clones encode a substantial portion of a methylenetetrahydrofolate dehydrogenase. These sequences represent the first corn, rice and soybean sequences encoding methylenetetrahydrofolate dehydrogenase.

Paragraph at page 25, lines 7-29:

A chimeric gene comprising a cDNA encoding the instant polypeptides in sense orientation with respect to the maize 27 kD zein promoter that is located 5' to the cDNA fragment, and the 10 kD zein 3' end that is located 3' to the cDNA fragment, can be constructed. The cDNA fragment of this gene may be generated by polymerase chain reaction (PCR) of the cDNA clone using appropriate oligonucleotide primers. Cloning sites (NcoI or SmaI) can be incorporated into the oligonucleotides to provide proper orientation of the DNA fragment when inserted into the digested vector pML103 as described below. Amplification is then performed in a standard PCR. The amplified DNA is then digested with restriction enzymes NcoI and SmaI and fractionated on an agarose gel. The appropriate band can be isolated from the gel and combined with a 4.9 kb NcoI-SmaI fragment of the plasmid pML103. Plasmid pML103 has been deposited under the terms of the Budapest Treaty at ATCC (American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209), and bears accession number ATCC 97366. The DNA segment from pML103 contains a 1.05 kb SalI-NcoI promoter fragment of the maize 27 kD zein gene and a 0.96 kb SmaI-SalI fragment from the 3' end of the maize 10 kD zein gene in the vector pGem9Zf(+) (Promega). Vector and insert DNA can be ligated at 15°C overnight, essentially as described (Maniatis). The ligated DNA may then be used to transform *E. coli* XL-1 BLUE® ~~XL1-Blue~~ (EPICURIAN COLI® ~~Epicurian Coli~~ XL-1 BLUE® ~~Blue™~~; Stratagene). Bacterial transformants can be screened by restriction enzyme digestion of plasmid DNA and limited nucleotide sequence analysis using the dideoxy chain termination method (SEQUENASE® ~~Sequenase™~~ DNA Sequencing Kit; U.S. Biochemical). The resulting plasmid construct would comprise a chimeric gene encoding, in the 5' to 3' direction, the maize 27 kD zein promoter, a cDNA fragment encoding the instant polypeptides, and the 10 kD zein 3' region.

Paragraph at page 26, lines 11-24:

The particle bombardment method (Klein et al. (1987) *Nature* 327:70-73) may be used to transfer genes to the callus culture cells. According to this method, gold

particles (1 µm in diameter) are coated with DNA using the following technique. Ten µg of plasmid DNAs are added to 50 µL of a suspension of gold particles (60 mg per mL). Calcium chloride (50 µL of a 2.5 M solution) and spermidine free base (20 µL of a 1.0 M solution) are added to the particles. The suspension is vortexed during the addition of these solutions. After 10 minutes, the tubes are briefly centrifuged (5 sec at 15,000 rpm) and the supernatant removed. The particles are resuspended in 200 µL of absolute ethanol, centrifuged again and the supernatant removed. The ethanol rinse is performed again and the particles resuspended in a final volume of 30 µL of ethanol. An aliquot (5 µL) of the DNA-coated gold particles can be placed in the center of a KAPTON® ~~Kapton™~~ flying disc (Bio-Rad Labs). The particles are then accelerated into the corn tissue with a BIOLISTIC® ~~Biolistic™~~ PDS-1000/He Particle Delivery System (Bio-Rad Instruments, Hercules CA), using a helium pressure of 1000 psi, a gap distance of 0.5 cm and a flying distance of 1.0 cm.

Paragraph at page 27, lines 34-37:

Soybean embryogenic suspension cultures may then be transformed by the method of particle gun bombardment (Klein et al. (1987) *Nature* (London) *327*:70, U.S. Patent No. 4,945,050). A DUPONT® BIOLISTIC® ~~DuPont Biolistic™~~ PDS1000/HE Particle Delivery System instrument (helium retrofit) can be used for these transformations.

Paragraph at page 29, lines 6-21:

Plasmid DNA containing a cDNA may be appropriately digested to release a nucleic acid fragment encoding the protein. This fragment may then be purified on a 1% NUSIEVE® GTG® ~~NuSieve GTG™~~ low melting point agarose gel (FMC). Buffer and agarose contain 10 µg/ml ethidium bromide for visualization of the DNA fragment. The fragment can then be purified from the agarose gel by digestion with GELASE™ ~~GELase™~~ (Epicentre Technologies) according to the manufacturer's instructions, ethanol precipitated, dried and resuspended in 20 µL of water. Appropriate oligonucleotide adapters may be ligated to the fragment using T4 DNA ligase (New England Biolabs, Beverly, MA). The fragment containing the ligated adapters can be purified from the excess adapters using low melting agarose as described above. The vector pBT430 is digested, dephosphorylated with alkaline phosphatase (NEB) and deproteinized with phenol/chloroform as described above. The prepared vector pBT430 and fragment can then be ligated at 16°C for 15 hours followed by transformation into DH5® ~~DH5~~ electrocompetent cells (GIBCO BRL). Transformants can be selected on agar plates containing LB media and 100 µg/mL ampicillin. Transformants containing the gene encoding the instant polypeptides are

then screened for the correct orientation with respect to the T7 promoter by restriction enzyme analysis.

Paragraph at page 30, lines 17-37:

Purification of the instant polypeptides, if desired, may utilize any number of separation technologies familiar to those skilled in the art of protein purification. Examples of such methods include, but are not limited to, homogenization, filtration, centrifugation, heat denaturation, ammonium sulfate precipitation, desalting, pH precipitation, ion exchange chromatography, hydrophobic interaction chromatography and affinity chromatography, wherein the affinity ligand represents a substrate, substrate analog or inhibitor. When the instant polypeptides are expressed as fusion proteins, the purification protocol may include the use of an affinity resin which is specific for the fusion protein tag attached to the expressed enzyme or an affinity resin containing ligands which are specific for the enzyme. For example, the instant polypeptides may be expressed as a fusion protein coupled to the C-terminus of thioredoxin. In addition, a $(His)_6$ peptide may be engineered into the N-terminus of the fused thioredoxin moiety to afford additional opportunities for affinity purification. Other suitable affinity resins could be synthesized by linking the appropriate ligands to any suitable resin such as SEPHAROSE® 4B resin ~~Sepharose-4B~~. In an alternate embodiment, a thioredoxin fusion protein may be eluted using dithiothreitol; however, elution may be accomplished using other reagents which interact to displace the thioredoxin from the resin. These reagents include β-mercaptoethanol or other reduced thiol. The eluted fusion protein may be subjected to further purification by traditional means as stated above, if desired. Proteolytic cleavage of the thioredoxin fusion protein and the enzyme may be accomplished after the fusion protein is purified or while the protein is still bound to the THIOBOND™ ~~ThioBond™~~ affinity resin or other resin.

Title at page 34, line 2:

ISOLATED NUCLEIC ACIDS ENCODING METHYLENETETRAHYDROFOLATE DEHYDROGENASE ~~TETRAHYDROFOLATE METABOLISM~~ ENZYMES